STUDIES ON THE OOPLASMIC SEGREGATION IN THE EGG OF THE FISH, ORYZIAS LATIPES. III. ANALYSIS OF THE MOVEMENT OF OIL DROPLETS DURING THE PROCESS OF OOPLASMIC SEGREGATION

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Ooplasmic segregation, which generally occurs following fertilization in fish eggs, leads to the formation of the blastodisc. Studies of this movement have been done by Spek (1933; *Coregonus, Salmo,* etc.), Roosen-Runge (1938; *Brachydanio*), Lewis (1949; *Brachydanio*), and Costello (1948; *Nereis*), etc.

Although much attention has been paid to the protoplasmic movement in the yolk or endoplasmic region, observations on the movement in the cortical protoplasmic layer (cortex) have been restricted to the eggs of *Brachydanio* (Roosen-Runge, 1938) and of *Gasterosteus* (Thomopoulos, 1953), neither of which have oil droplets in the cortex. In *Oryzias* eggs, the protoplasm and yolk are well separated before fertilization and oil droplets are dispersed in the former. On fertilization, the oil droplets move toward the vegetal pole at a speed which can be measured accurately.

The present paper deals with analysis of the pattern of the movement of oil droplets, both natural and injected, during the formation of the blastodisc in *Oryzias* eggs.

Part I. Movement of Natural Oil Droplets

Methods

After fertilization in *Oryzias*, evenly dispersed oil droplets in the unfertilized egg cortex migrate toward the vegetal pole, fusing with each other, and finally assemble around the vegetal pole, turning the egg upside down within the chorion by buoyancy. Therefore, to prevent the rotation of the egg during observation, the egg must be placed with the animal pole down from the beginning, and fertilized; it is photographed simultaneously from the animal, vegetal and lateral sides at intervals of two minutes. The photographs of the egg are magnified 100-fold and superimposed to trace the movement of the oil droplets.

In the polar views, the distance along the curved surface between the oil droplets and the animal or vegetal pole is calculated from the tracings of the photographs. In the side view, the equator of the egg is taken as a reference line and the distance of the oil droplets from the line is calculated. Since the tracings of the moving oil droplets are almost parallel to the longitude of the egg, sidewise shifts of the droplet are neglected (Fig. 1).

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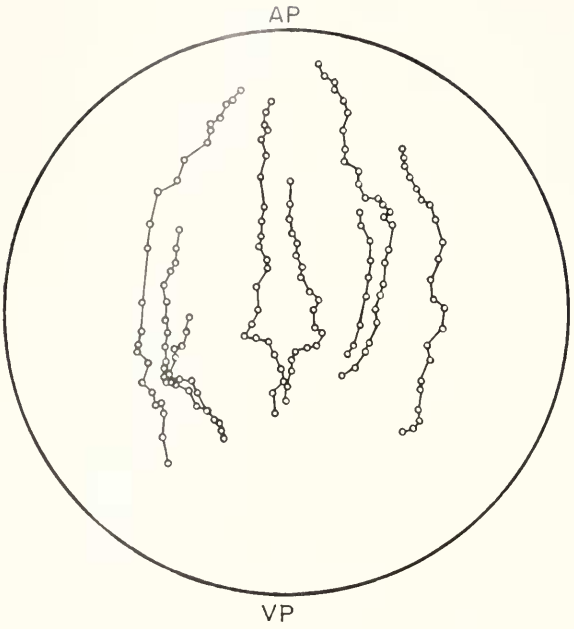

FIGURE 1. Tracings of moving oil droplets during ooplasmic segregation. AP: animal pole; VP: vegetal pole.

In the calculations, the egg is considered as a sphere. Since the egg is not strictly a sphere but rather an ellipsoid of revolution, the error coming from the approximation must be determined. In Figure 2, S is a sector of a sphere and E is that of an ellipsoid of revolution, a and b being the major and the minor axes of the

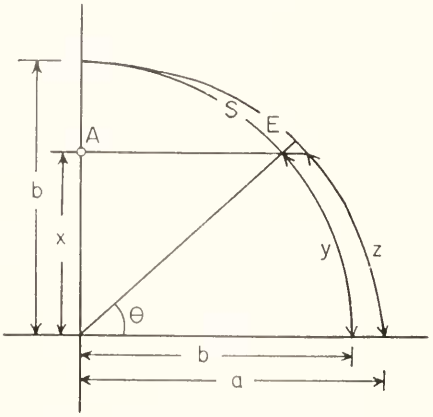

FIGURE 2. Procedure to obtain the actual distance or the angle θ from an apparent distance on the surface of sphere and of ellipsoid of revolution. S: a sector of a sphere; E: a sector of an ellipsoid of revolution; A: position of an oil droplet; a: major axis of ellipsoid of revolution; b: minor axis of the ellipsoid of revolution; x: apparent distance of an oil droplet from the reference line (equator); y: actual distance on the sphere; z: actual distance of x of the ellipsoid of revolution; θ: the angle at the center of the sphere embracing y.

latter. "A" represents the position of an oil droplet under observation and x is its apparent distance from the reference line, as expressed in an angle at the center embracing x; y is an arc of the circle and z is a section of the ellipsoid, which are the actual distances along the curved surfaces corresponding to the apparent distance x of the sphere and the ellipsoid of revolution, respectively. In other words, the aim of the calculation is to obtain either y or z from x.

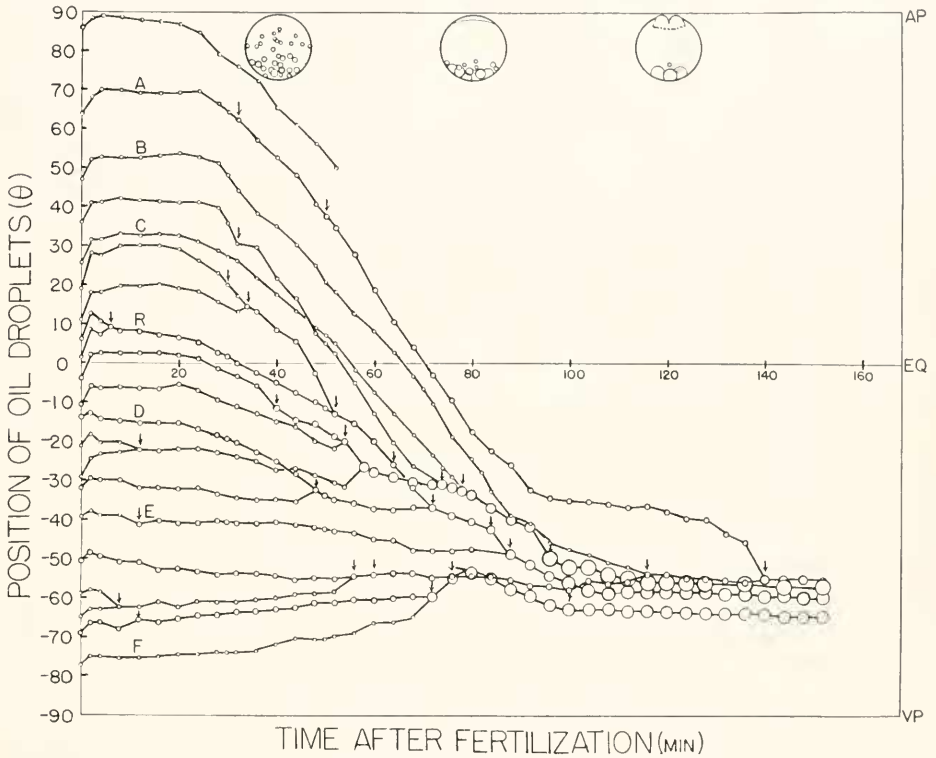

FIGURE 3. Time courses of the migration of oil droplets in terms of θ (22° C.). Ordinate: the value of θ, taking an equator as 0; abscissa: time after fertilization in minutes. AP: animal pole; VP: vegetal pole; EQ: equator; arrow: fusion of oil droplets. Alphabetic designation of the curves is for use in Figure 4.

In the *Oryzias* eggs used for the present measurements, the deviation of the ellipsoid from the sphere, a−b/a, is not larger than 0.08. For simplification of the calculation, a−b/a is taken as 0.10. On the basis of these figures, the deviation of z from y, z−y/z, turns out to be less than 0.03, which means that the error involved in the approximation as a sphere is negligible. The measurement for the region above $\sqrt{3}/2$ b (60° in θ) is supplemented by the measurements in the polar views.

Since an egg can be treated as a sphere, distances through which the oil droplets move can best be expressed in θ because θ is independent of individual fluctuation in the egg size.

Results

Figure 3 compares the time course of the migration in θ of oil droplets initially located at different regions of the egg. Within 2–4 minutes after fertilization, almost all the oil droplets shift transiently toward the animal pole to some extent (see the left end of Fig. 3). This shift is rather difficult to discern unless one is aware of this phenomenon beforehand. During this period, a decrease in the egg volume takes place in *Oryzias* as the result of the breakdown of cortical alveoli (T. Yamamoto, 1940). However, since the distance is expressed in θ, the decrease in the volume does not affect the measurement as long as the shrinkage occurs uniformly. Correspondingly to this stage when the animal region of the *Oryzias* egg is seemingly

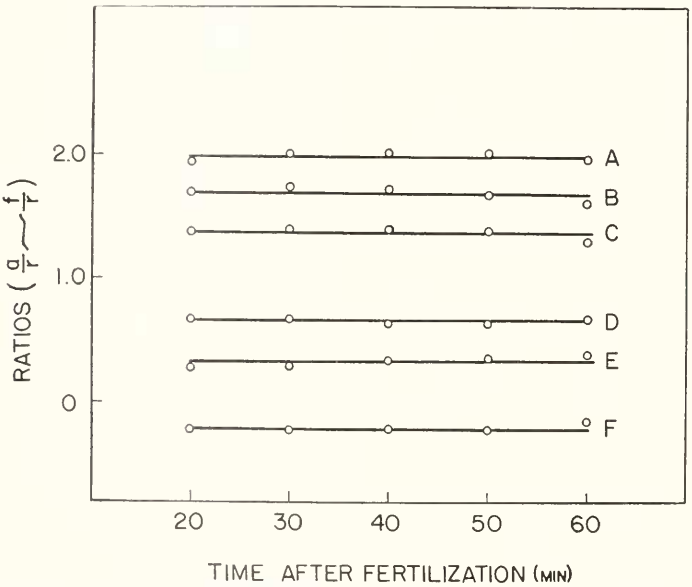

Figure 4. The ratio between the distance in θ of the reference oil droplet (R) from the base line ($-60°$) and the distance of a given oil droplet (A, B, C, D, E and F) from the line ($-60°$) at specified moments (a/r, b/r, c/r, d/r, e/r and f/r). Ordinate: ratios a/r, b/r, c/r, d/r, e/r and f/r; abscissa: time after fertilization.

contracting. Roosen-Runge (1938) describes, in *Brachydanio* eggs, an increase of the egg diameter and flattening of the animal pole region, as observed from the upper side. According to the present writer's observations of *Brachydanio* eggs made at this stage from the side, the egg flattens and so does the blastodisc region. Whether or not this flattening of the blastodisc region means a contraction at the animal region cannot be said at present.

In *Oryzias*, the oil droplets remain stationary thereafter for about 20 minutes, after which the movement of the oil droplets is resumed and they move toward the vegetal pole. This movement is particularly striking on the animal half.

On the other hand, the vegetal oil droplets, originally located within 30° in θ from the vegetal pole or beyond $-60°$ on the ordinate of Figure 3, continue to move toward the animal side even after the transient shift is over. Consequently, all the

droplets are assembled at the latitude of about $-60°$ as a ring. It must be mentioned that the ring eventually reaches the vegetal pole after several hours, by the morula stage (omitted from Fig. 3).

If the migration speeds of the oil droplets starting from various levels of the egg are compared at various moments after fertilization, it can be said that the higher the curve, the steeper the inclination, which means that the closer an oil droplet is to the animal pole, the faster it moves.

Next, the latitude of $-60°$ to which the oil droplets gather is taken as a base line, and the positions of seven droplets, A, B, C, D, E, F and R (Fig. 3) from the new base line are read in θ (a, b, c, d, e, f and r) for 20, 30, 40, 50 and 60 minutes after fertilization. In Figure 4, the ratios a/r, b/r, c/r, d/r, e/r and f/r at the specified moments are plotted. As is clear, the ratios for respective droplets remain almost unchanged during the migration. This means that these droplets approach the base line at a speed proportional to the original distance of the oil droplet from the base line, theoretically reaching the base line simultaneously, which is more or less what is observed.

The tracings of the oil droplets look as though the droplets might be attached to a stretched rubber membrane and carried toward $-60°$ in θ by the snapping of the membrane, as the result of breaking at the two poles.

As is well known, the oil droplets frequently fuse with each other during the migration. When this happens, the speed of the fused droplet comes close to that of the slower or larger partner (see Fig. 3).

Discussion

Transient shift of oil droplets toward the animal pole immediately following activation

From the previous study by the author (Sakai, 1961), the unfertilized egg of *Oryzias*, deprived of the chorion, is flattened when observed from the side. On fertilization or activation, the egg is further flattened in the region where the cortical response is taking place. This flattening (decrease of the tension at the surface) spreads from the activated point with the wave of breakdown of cortical alveoli. After 2–4 minutes, when the cortical change is almost completed, the egg begins to bulge again from the activated point (Sakai, 1961).

In the fertilized egg enclosed within the chorion, while the tension at the surface is decreasing near the animal pole, the egg cortex on the vegetal side must still be adhering to the chorion because the cortical response has not yet taken place there. By the time the egg cortex at the vegetal pole detaches itself from the chorion with decreased tension, tension near the animal pole should have already begun to increase. If so, the egg cortex on the vegetal side must be pulled toward the animal pole and the transient shift of oil droplets toward the animal pole, mentioned in connection with Figure 3, becomes understandable.

Stationary phase

Within about 20 minutes after the completion of the cortical response, the naked egg bulges higher than before fertilization. This 20-minute period coincides with that of the stationary phase, so that it seems that oil droplets do not begin to move

until the tension at the surface reaches a certain level. Similarly, if a part of the yolk is sucked out from the egg about 15–20 minutes after fertilization, when oil droplets would begin to migrate under natural conditions, the egg is flattened and the droplets in the treated egg do not migrate until the egg rounds up again. In such eggs having lost a part of the yolk, the formation of the blastodisc tends to be retarded and so does the accumulation of oil droplets at the vegetal pole. The recovery of the egg shape (recovery of the level of tension), therefore, seems to be essential for the initiation of the migration of oil droplets. These observations are of interest in connection with the fact that, in *Brachydanio* eggs, a protoplasmic movement inside the yolk and a counterstream in the protoplasmic coat begin only after the egg becomes exactly round (Roosen-Runge, 1938). However, no explanation is available concerning the manner in which a higher membrane tension and the bipolar segregation of the protoplasmic components are connected with one another.

Part II. Movement of Injected Oil Droplets Following Activation

After analyzing the movement of the natural oil droplets, it is of interest to see how a droplet of oil foreign to the egg will move when it is introduced into the egg by injection.

Methods

Salad oil (as a neutral oil; acid value (A.V.) = 0.22), liver oil (as an acidic oil; A.V. = 0.52), and mineral oil (as a non-polar oil) were used as substances to be injected.

To distinguish the injected oil droplet from the natural ones of the eggs, the oil to be injected was previously stained with Sudan III. By using a micromanipulator, an oil droplet of a size similar to that of natural ones (20–70 μ in diameter) was injected into the cortical protoplasmic layer (cortex) of the unfertilized eggs, either centrifuged or non-centrifuged, and of the fertilized eggs at the one-, two-, and 8-cell stages. When oil droplets are injected into the fertilized eggs, the chorion is previously removed by using the hatching enzyme of *Oryzias* (Sakai, 1961). Since the cortex of the unfertilized eggs is very thin, the tip of the injection needle sometimes misses it. If the oil happens to be injected into the yolk, the oil moves freely by gravity. If the oil is injected at too shallow a layer, the oil is apt to be squeezed out of the egg surface into the perivitelline space after the alveolar breakdown. As a result, successful injection can easily be determined.

For measuring the movement of the injected oil droplet, the same procedure is applied as that which was used in Part I.

Results and Conclusion

Behavior of the injected oil droplet

The oil of *Oryzias* eggs is a kind of neutral oil because it is stained deeply with Sudan III and Sudan black, and also stained a pink color with Nile blue sulfate at about pH 7.0. Such a stainability is the same as that of oil droplets of *Onchorynchus* eggs (K. Yamamoto, 1958).

To test another kind of neutral oil, salad oil is used. After the injection of salad oil, the eggs are fertilized or activated by pricking. Although some eggs are activated by the injection procedure itself, the behavior of the injected oil is much the same as that in the eggs activated after a successful injection in an inactivated condition.

When injected at the equatorial region, the injected oil, on pricking, generally migrates toward the vegetal pole, fusing with the natural oil during the movement (Fig. 5). In fertilizable but slightly under-ripe eggs, merging of the natural oil droplets among themselves rarely occurs but even under such a circumstance, the injected oil droplets move to the pole side by side with the natural ones. Occasionally, the injected oil fails to move, probably owing to imperfection of injecting technique, in spite of the successful migration of natural oil droplets lying closer to the animal pole than the injected one. In such cases, natural droplets situated

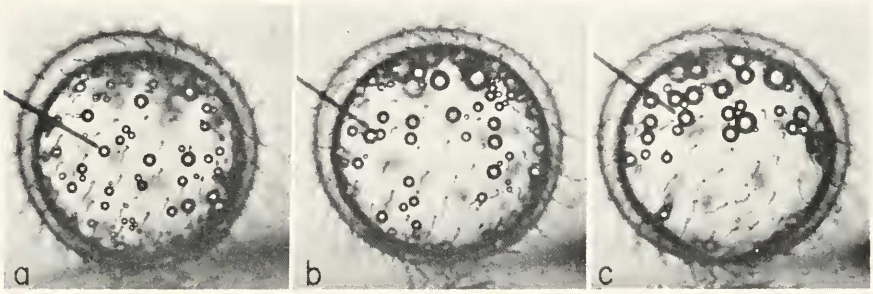

FIGURE 5. Movement of oil droplet injected at the equatorial region of the unfertilized egg (side view). The egg is placed upside down to avoid the rotation of the egg caused by assembling oil droplets. (a) 20 minutes after fertilization; (b) about 40 minutes after fertilization; (c) one hour after fertilization. Injected oil has fused with the natural oil.

on the animal half overtake the injected oil and pass it closely around its circumference. However, if the oil droplet migrates at all, it always migrates toward the vegetal pole, and never toward the animal pole. Quantitatively, too, the behavior of the injected oil corresponds well to that of the natural droplets as shown in Figure 6. The frequency of the migration is less when the oil is injected close to the animal pole than when injected at the equator.

To clarify whether or not the behavior of the injected oil varies with its properties, similar experiments were repeated by using liver oil, mineral oil and the oil of *Oryzias* eggs. These experiments give substantially the same results as that of the salad oil. Judging from the fact that the injected oil of *Oryzias* itself sometimes fails to move, it is most likely that the failure is not due to the properties of the oil but to disturbance caused by the injection technique.

Relationship between movement of protoplasm and oil droplets

From the foregoing results, the oil droplets migrate irrespective of the nature of the oil itself. However, this still leaves the possibility open that the migration of the oil droplets is somehow coupled with the movement of the protoplasm. To make sure of this point, the following two conditions were tested: (1) weakly centrifuged

eggs (100–200 *g* for about three minutes), with the natural oil droplets shifted to one side but leaving the protoplasm undisturbed, (2) strongly centrifuged eggs (900–1800 *g* for 10 minutes), with both oil and protoplasm localized at the opposite sides.

In non-injected eggs, shifting of the natural oil by weak centrifugation does not interfere with ooplasmic segregation, regardless of the abnormal localization of

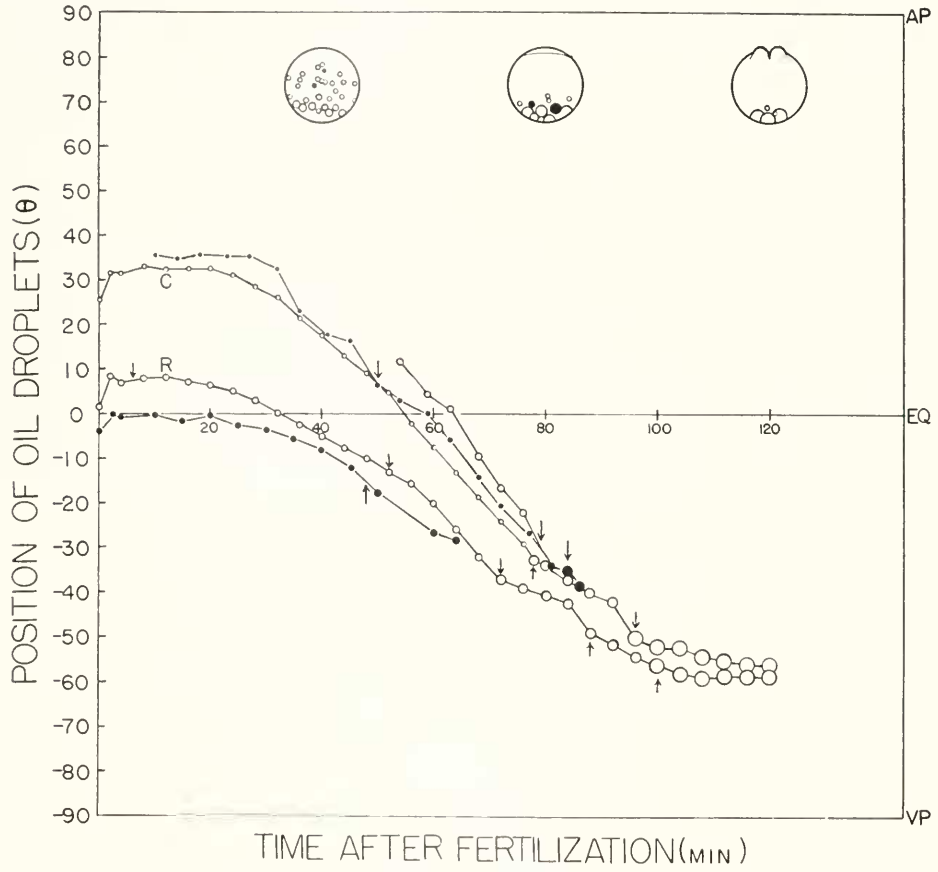

FIGURE 6. Time courses of the migration of injected oil droplets in comparison with the natural droplets. Ordinate: the value of θ, taking the equator as 0; abscissa: time after fertilization in minutes; closed circle: injected oil droplets; open circles: natural oil droplets; AP: animal pole; VP: vegetal pole; EQ: equator; arrow: fusion of oil droplets.

the droplets after centrifuging. In centrifuged eggs, the injection is made where the natural oil droplets are no longer found, although protoplasm and cortical alveoli are still present in the egg cortex. In spite of the absence of the natural oil droplets around the injected oil, it can migrate toward the vegetal pole all by itself.

On the other hand, by strong centrifugation, the protoplasm of the unfertilized egg can be shifted in the animal region and the oil is massed at the vegetal pole by orienting the egg by a capillary tube. As the result, a blastodisc is formed at the

centrifugal animal side where the protoplasm has already collected. The oil is injected at the equatorial region where little protoplasm is found. Under these conditions, the injected oil is fixed at the injected point and never migrates toward the vegetal pole within the observation period of three hours.

To further confirm the idea that the migration of oil droplets is caused by the movement of the protoplasm, the oil is injected near the equator of the egg at the one-, two- and 8-cell stages in which the protoplasmic segregation has almost been completed. The injected oil droplets never migrate toward the vegetal pole. The relationship between oil and protoplasm is also pointed out by the following results.

When an egg is forced into a capillary, both the migration of oil droplets and the formation of the blastodisc are much delayed. Furthermore, if more than one protoplasmic accumulation is induced by polyspermy or strong prickings, such protoplasmic accumulations are always accompanied by the migration of oil droplets toward the opposite side of each accumulation (Sakai, 1964a). Further, the experiments on partial activation indicate that the oil migration does take place only in the activated half (Sakai, 1964b).

If the migration of the protoplasm has a leading role over the movement of natural oil droplets, the elimination of the oil droplets is expected to have no influence on the movement of the remaining protoplasm. In the eggs weakly centrifuged at 100–200 *g* for about 5 minutes just after the fertilization, the cortex protrudes where the oil is forced to gather. Such a mass of oil can be sucked out with a micropipette. As in *Nereis* egg fragments observed by Costello (1940), yet the migration of the protoplasm can still occur and form the blastodisc.

On the other hand, careful observation reveals that the protoplasmic movement always precedes that of the oil droplets, that is, by the end of the stationary phase, a small amount of protoplasm has already begun to accumulate at the animal pole, slightly flattening the yolk surface under it.

Considering the above-mentioned results in connection with this observation, it will be concluded that the migration of oil droplets is a consequence of the movement of protoplasm toward the animal pole.

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Summary

The movement of oil droplets in *Oryzias* eggs, natural and artificially injected, was analyzed during ooplasmic segregation. (a) During 2–4 minutes after fertilization, natural oil droplets are shifted transiently toward the animal pole, followed by a stationary phase of about 20 minutes. After this phase, all of the oil droplets coming either from the animal or from the vegetal side assemble at about −60° below the equator as a ring and later reach the vegetal pole. The migration is faster in droplets coming greater distances than in those coming shorter distances. (b) The pattern of the migration of injected oil droplets is the same as that of the natural ones, irrespective of their nature. The migration is possible in weakly centrifuged eggs in which the protoplasm remains undisturbed in the cortex.

However, injected oil droplets no longer move after shifting of the protoplasm by strong centrifugation or after the completion of ooplasmic segregation.

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